

**MULTIPLE MATING IN *XIPHOPHORUS BIRCHMANNI*, A LIVE-
BEARING SWORDTAIL FISH**

A Senior Scholars Thesis

by

COURTNEY PASSOW

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biology

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ABSTRACT

Multiple Mating in *Xiphophorus birchmanni*, a Live-bearing Swordtail Fish.
(April 2011)

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Male and female mating behaviors have been extensively studied in *Xiphophorus birchmanni*; however the genetic mating system has not been characterized in this species. *X. birchmanni* is a live-bearing fish, so females can be easily collected with their young. Gravid females were collected from two populations of sheepshead swordtail, *X. birchmanni* in the state of Hidalgo, Mexico. To determine if female phenotype is related to number of mates, we examined female body size, number of offspring, and number of mates. We used four polymorphic microsatellite markers to genotype 40 wild caught *X. birchmanni* females and their offspring. COLONY was then used to assign parentage and estimate the rate of multiple mating (Wang 2004). From our analysis, we can conclude that *X. birchmanni* females mate multiply at rates between one and eight male sires per brood. There was also a positive correlation between the number of mates and size of the females, as well as the number of offspring per brood and size of the females. Interestingly, *X. birchmanni* females have a higher average rate

of multiple mating compared to other species in this genus. These results suggest that sexual selection may be acting very differently in *X. birchmanni* compared to congeneric species. In particular, the high rate of multiple mating by females may open the door for post-copulatory sexual selection to play a significant role in determining reproductive success.

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I would also like to acknowledge my parents for the support and love that they have provided me throughout my college career. If not for them, I would not have had the motivation and dedication to attend college as a biology student and now graduate with the plans of attending a graduate program in my field of study.

Although they did not help me with my actual project, I would like to thank the graduate students in our lab that supported both me and other students with guidance and support. I feel that without their help, I would have given up on this project especially when I hit the rough points where things just did not want to work.

I would also like to acknowledge the undergraduates who worked in Dr. Rosenthal's lab. Although they did not help with the project, they would help with editing the paper and give me emotional support when things got rough and my project hit snags.

Last, but certainly not least, I would like to acknowledge the undergraduate student, Lauren McMahon, who helped me set up plates when I was occupied with other tasks. Not only was she helpful and always there when I needed her, but she grasped the concepts quickly and was a big help to me, as well as to my ability to complete the project on time.

NOMENCLATURE

μl	Microliters
Bp	Basepairs
DNA	Deoxyribonucleic Acid
DTP	Dithiothreitol
Ng	Nanogram
PCR	Polymerase Chain Reaction
ProK	Proteinase Kinase
Rpm	Rotations per minute
TE Buffer	Tris\EDTA buffer

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CHAPTER I

INTRODUCTION

Male and female mate preference has been extensively studied throughout the *Xiphophorus birchmanni* species. However, there has been little done to characterize the genetics of the mating system. We are using the swordtail species to study mating preferences in females to help us understand the role of preference in mating success, which will affect evolution of the species (Andersson 1994). Swordtails were chosen as the model organism in this study due to the females being live-bearing, allowing us to collect both the offspring and gravid females with ease in the wild. Due to the fact that female and male swordtails may have different mating preferences in the swordtail population, it is important to isolate the male and female preferences. Male swordtail fish seem to prefer to mate with larger females, since larger females will be able to hold more embryos and produce more offspring, increasing the male's overall fitness. Alternatively, since there is no parental care in the swordtail species, it is suggested that females seem to want to mate with multiple males, attempting to increase their offspring's chance of survival (Simmons et al. 2008). Due to the conflict of interests between the males and females in this species, the eventual mating success of males is not easy to predict. For example, we might expect that small females will prefer to mate with multiple males, but since the males prefer to mate with larger females, the

This thesis follows the style of Evolution.

small female may not succeed. Large females could also prefer mate multiply or mate with a certain phenotype, causing males with certain phenotypes less of a chance to succeed (Fisher et al. 2009).

Motivated by this problem, we aim to explore the role females play in mating success, particularly the number of male sires for each female brood. Our null hypothesis is that preference does not actually affect mating success; mate number is unrelated to female characteristics. An alternative hypothesis is that females choose and prefer more mates. If this is the case, then females in the swordtail population would have multiple mates, but would have no bias towards ornamentation on the males; every male would sire an equal proportion of the brood. Another alternative hypothesis is that females mate multiply but that male fertilization success is biased, either because of cryptic female choice or male sperm competition. This pattern could arise if the female has a preference for more ornamented mates, or if males differ in their ability to fertilize successfully. In this case, we would see multiple mating but that one male has higher fertilization success. Finally, it could be that males prefer large females. If this is the case, then larger females in *X. birchmanni* would have higher rates of mating multiply than smaller females in the population after controlling for brood size.

Although the mating system of *X. birchmanni* has not been genetically characterized, there are other species in the *Xiphophorus* genus that have been studied. *Xiphophorus helleri* and *Xiphophorus multilineatus* are two species in the genus that have been

analyzed and found to be multiply mating (Simmons et al 2008 and Luo et al. 2005). With our data we hope to compare the number of mates between our species and the other species to determine if the pattern of multiple mating is comparable.

Over the course of a year, we investigated the relationship between female body size, number of mates and the number of offspring in the sheepshead swordtail (*Xiphophorus birchmanni*). Although females may mate with multiple males, mating doesn't guarantee fertilization of the embryos. Therefore, microsatellite markers were used on both the females and the embryos to determine the number of mates each female had in a wild population of swordtails. From the sheepshead swordtail collected, we planned to use between 35 to 40 females from two populations. The embryos from each female were genotyped. Each female produced 10 to 30 progeny. By using the microsatellite markers on the female fin clips and embryos, while including the analysis of the phenotype of the female, we determined the relationship between the female's size, the number of mates and offspring the female had.

CHAPTER II

METHODS

Sample collection

The *Xiphophorus birchmanni* females and embryos were collected from two locations throughout Calnali, Mexico in the state of Hidalgo. The collection sites were given names based on the towns near them. These locales were termed San Pedro and Coacuilco. 23 gravid females were collected from San Pedro and 11 gravid females were collected from Coacuilco. They were then preserved in ethanol and later dissected in the laboratory to remove both a fin clip and embryos from the females. Upon analysis, the embryos were taken out of the ethanol and through dissection, were removed from the yolk sac attached during development.

DNA extraction

Once the embryos were removed from the yolk sac, they and the female fin clips were separated and put into small microcentrifuge tubes with a solution containing 20µl of DTP, 7µl of ProK, and 600µl of cell lysis. Once placed in the microcentrifuge tubes and then capped, the samples were wrapped in Saran wrap, and then put at in the incubating mini shaker at a temperature of 54 °F rotating at 160 rpm overnight. The following morning, the samples were removed from the incubating mini shaker and allowed 10-15 minutes to cool. Once cooled, protein precipitation was then added to each sample, recapped and then placed in the centrifuge for 5 minutes at approximately 12,000-13,000

rpm at a temperature of 4 °C. After samples were removed, the supernatant was poured into new microcentrifuge tubes containing 600µl of chilled isopropanol. The samples were then inverted 50 times, and placed into the centrifuge where they spun for 10 minutes at 12,000-13,000 rpm. The temperature was not required to be monitored during this step. Once the samples had been spun, they were removed from the centrifuge and the supernatant was discarded while leaving the pellet that was formed during the spin cycle in the bottom of the microcentrifuge tubes. This pellet contains the DNA from the fin clip or embryo extracted into a small compact form. 600µl of 70% ethanol was then added to the microcentrifuge tubes to “wash” the pellet for cleaner results upon analysis. The tubes were then individually inverted by hand 3-4 times and then placed into the centrifuge again for 10 minutes spinning at 12,000-13,000 rpm. Once removed from the centrifuge, the supernatant was again discarded while keeping the pellet at the bottom of the tube. During this step, it was important to remove any excess supernatant to dry the pellets. The pellets inside the microcentrifuge tubes were allowed to dry for 30 minutes to an hour. Once the pellets were dry and all excess liquid evaporated, 52µl of TE buffer was added and the samples were then stored in a 4°C refrigerator.

DNA quantification

At a later date, the samples were taken from the refrigerator to a Nano Dropper to obtain the concentration of DNA (obtained from the pellet) in each sample. 1.5µl of each DNA sample was placed onto to the Nano Dropper and the value obtained was recorded in a data sheet. Once the value was obtained, each sample was diluted to 40 ng/µl so that

there was consistency in the data sets. The samples that had values lower than 40ng/μl were not diluted and used directly in the PCR plates.

Polymerase chain reaction (PCR)

Once all the samples had been diluted to 40ng/μl, sets of 96 samples were run through PCR methods with four different microsatellite markers. The steps during PCR depended on which microsatellite marker was being set up. The microsatellite markers used in this experiment were Msd 072, Msd 036, Msd 029, and Msd 049. The procedures for each of them were as followed.

Msd 072

With primer Msd 072; 8.336μl of distilled water, 1.5μl PCR buffer, 1.5μl of 2μM dNTP, 1.2μl 25mM MgCl₂, 0.22μl of Msd 072 Forward primer, 0.22μl of Msd 072 Reverse primer, and 0.144μl of TAQ polymerase was added into each well of a 96 well plate. The program used on the thermal cycler were as followed for Msd 072; step one was 94°F for 2:00 mins, step two was 94°F for 0:15secs, step three 60°F for 0:30secs, step four 72°F 1:00 min, step five went back to step two through five and repeated 35 times, and step six was 72°F for 4:00 minutes.

Msd 049

With primer Msd 049; 8.036μl of distilled water, 1.44μl PCR buffer, 1.44μl of 2μM dNTP, 1.5μl 25mM MgCl₂, 0.22μl of Msd 049 Forward primer, 0.22μl of Msd 049

Reverse primer, and 0.144 μ l of TAQ polymerase was added into each well of the plate. The program used on the thermal cycler were as followed for Msd 049; step one was 94°F for 2:00mins, step two was 94°F for 0:15secs, step three 60°F for 0:30secs, step four 72°F 1:00min, step five went back to step two through five and repeated 35 times, and step six was 72°F for 4:00minutes.

Msd 036

With primer Msd 036; 5.736 μ l of distilled water, 1.44 μ l PCR buffer, 1.44 μ l of 2 μ M dNTP, 1.8 μ l 25mM MgCl₂, 0.22 μ l of Msd 036 Forward primer, 0.22 μ l of Msd 036 Reverse primer, and 0.144 μ l of TAQ polymerase was added into each well of the plate. The program used on the thermal cycler was as followed for Msd 036; step one was 94°F for 2:00mins, step two was 94°F for 0:15 secs, step three 58°F for 0:30secs, step four 72°F 1:00 min, step five went back to step two through five and repeated 35 times, and step six was 72°F for 4:00 minutes.

Msd 029

With primer Msd 029; 8.456 μ l of distilled water, 1.44 μ l PCR buffer, 1.44 μ l of 2 μ M dNTP, 1.2 μ l 25mM MgCl₂, 0.16 μ l of Msd 029 Forward primer, 0.16 μ l of Msd 029 Reverse primer, and 0.144 μ l of TAQ polymerase was added into each well of the plate. The program used on the thermal cycler was as followed for Msd 029; step one was 94°F for 2:00mins, step two was 94°F for 0:15 secs, step three 58°F for 0:30 secs, step four

72°F 1:00 min, step five went back to step two through five and repeated 45 times, and step six was 72°F for 4:00 minutes.

Other than marker Msd 036 which contained 4µl of DNA and 11µl of component mixture, the other three primers contained 2µl of DNA and 13µl of component mixture.

Fragment analysis

After all four markers were amplified and multiplexed together; a 96 well plate was acceptable for shipment if a minimal of 80-85% of the overall plate had amplified correctly. A gel was constructed and run to ensure that each well in the 96 well plates amplified correctly. This was verified before sending the plate off for fragment analysis, to make sure that there was amplified product which would yield results, rather than sending off product that was not amplified correctly. Once each microsatellite marker had been confirmed to contain amplified DNA, the four markers were multiplexed together in a 96 well half skirted plate. Each well in the plate contained 13.8µl of HiDi, 0.2µl of liz size standard, and 1µl of the combined DNA from each of the four microsatellite plates run through the PCR methods diluted with 1µl of water. The plates were then sent out for fragment analysis to Cornell University CLC DNA analysis center.

After the results from the fragment analysis returned from Cornell University, they were then analyzed using a computer program, Peak Scanner. Peak Scanner displays the

peaks for each microsatellite marker and allows the user to determine the size or base pairs of each peak. Each peak averaged between 130-300bp, making it simple to distinguish between what was actually the amplified DNA and what was just “background noise.”

Parentage analysis

Once all of the peaks have been read, the data is entered into Microsoft Excel, and then the collected peak results binned to combine the data and format it for the parentage program, COLONY (Wang 2004). After the data has been binned, the alleles must be tested to determine if they are in Hardy-Weinberg Equilibrium, indicating that they are neutral. The program GENEPOP was used to assess if the four microsatellite markers used were in Hardy-Weinberg Equilibrium. This is to ensure that there is no selection occurring where the microsatellite markers are annealing.

The binned fragment analysis data results are then run through the program COLONY to determine if the females had been mating multiply (Wang 2004). This program will show how many mates each female had and which embryos are sired by different fathers. Since the father’s genotype is unknown, it will also give us a hypothesized genotype for the father and an ID number to distinguish between the different fathers.

Once the analysis was completed using the program COLONY, each of the offspring were then analyzed to make sure that they paired up correctly with their maternal

genotype. The ones incorrectly paired were removed from the final analysis. Once these samples were removed, the maternal genotype and offspring genotype were used to determine the father's genotype, and how many sires each female had per brood.

CHAPTER III

RESULTS

Data was collected on the female body size (overall length of each female in mm), number of offspring or size of brood, and how many mates each female had. Figure 1 shows the relationship between the number of offspring each female had and the size (length (mm)) of each female used for the paternity analysis. This was used to determine if a correlation existed between the number of offspring and the overall size of the female

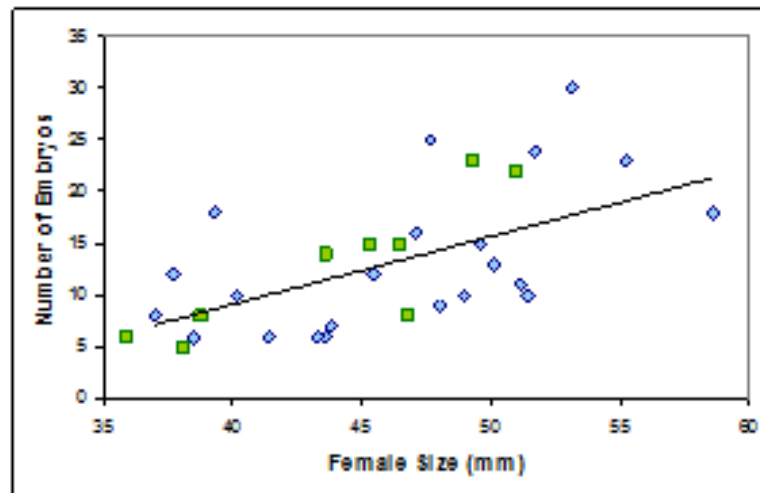


Figure 1. Female Size and number of offspring.

Larger females in San Pedro (blue) and Coacuilco (green) have larger broods in both populations ($R^2 = 0.394142$, $p = 0.0002$).

Upon running GENEPOP, it was concluded that for the Coacuilco samples, all of the microsatellite markers were in Hardy-Weinberg equilibrium with p-values greater than 0.05. However, two of the four microsatellite markers in San Pedro were not in Hardy-Weinberg equilibrium ($p < 0.05$). The reason for this was there was heterozygous excess within the population which was causing the samples to be out of Hardy-Weinberg. Possible reasons for heterozygous excess will be discussed in the conclusions. However this did not affect our analysis because we are not assessing population genetic structure. Figure 2 shows the relationship between the two populations collected for analysis, Coacuilco and San Pedro.

Females in both populations mate multiply (Figure 2). In San Pedro, females mate with between one and eight mates ($\bar{X} = 3.95$). In Coacuilco, females mated with between one and four mates ($\bar{X} = 2.81$). Although San Pedro seems to have a higher rate of multiple mating, this difference is not statistically significant ($p = 0.08$), and may only reflect differences in sample size.

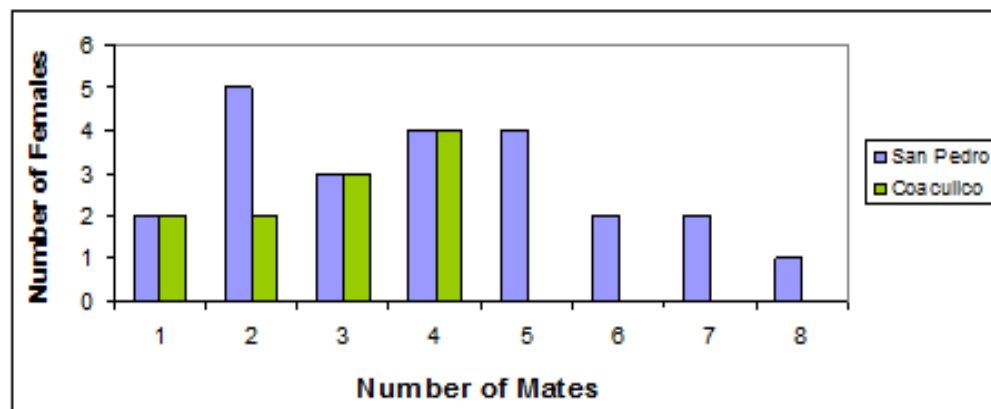


Figure 2. Number of sires per brood per population.

Once the number of males per female brood was obtained, the male mates and female size were analyzed to see if there was a correlation between female size and the number of male mates the female had. This can be seen in Figure 3. The graph of number of mates vs. female body size shows that there is a positive correlation between the size of the female and the number of mates she mated with.

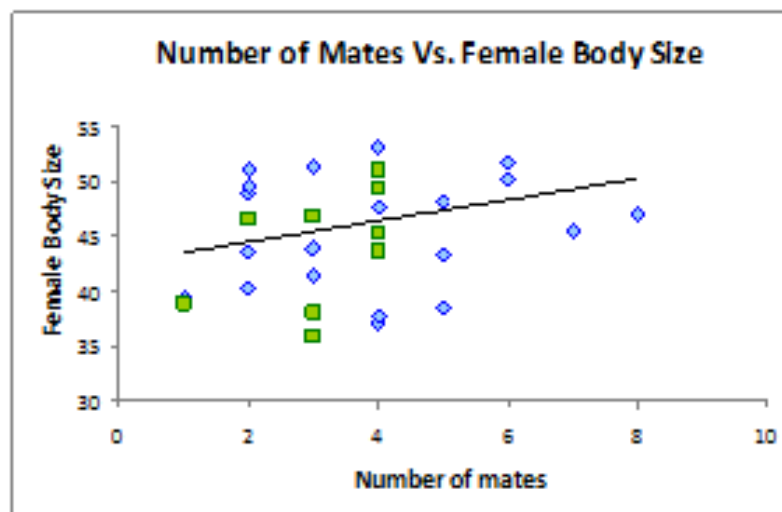


Figure 3. Number of mates vs. female body size.

Larger females in both San Pedro (blue diamonds) and Coahuilco (green squares) have more mates ($R^2=0.134417$, $p=0.0425$).

Although there was obvious multiple mating in both *X. birchmanni* populations there was a noticeable difference between the sires in each brood. As figures 4a – 4d show, there was a dominant male observed in each brood, however levels of males dominance were not as high in 4b and 4c. Other males that helped sire the female's brood did not have as many offspring as the first male, decreasing their overall fitness within the

brood. Each brood had a dominant male which sired the most offspring while the lesser males usually sired between one and three offspring per brood.

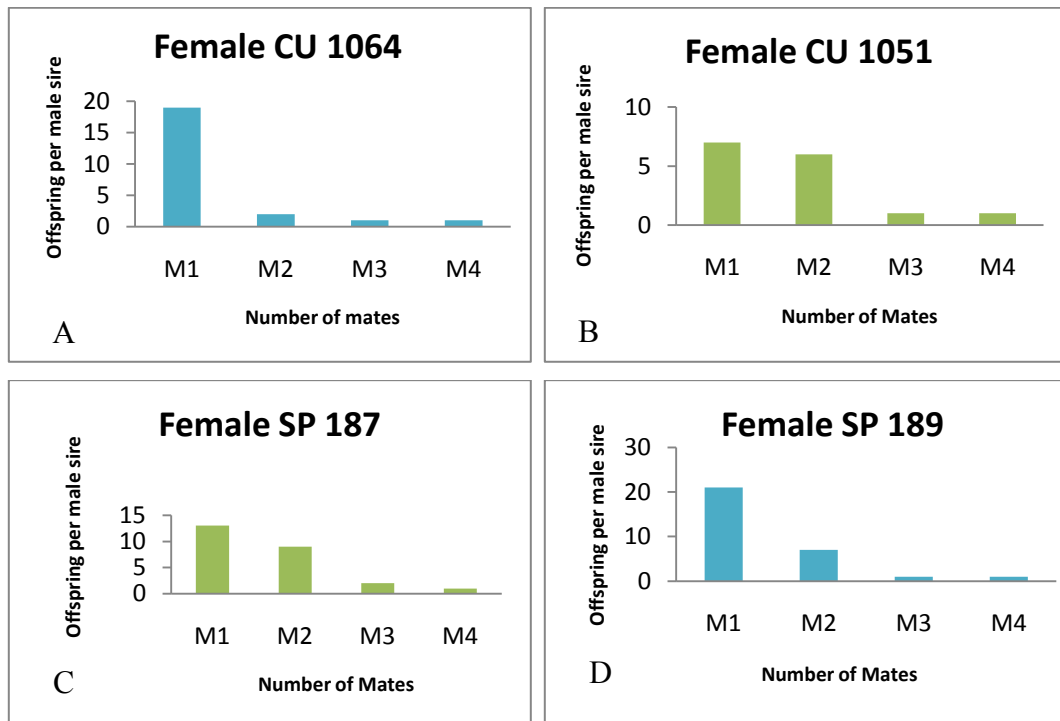


Figure 4. (A-D) Number of mates vs. number of offspring.

Possible causes for problems in this experiment were the collected samples of the embryos and the female fin clips. Many of the embryo samples had been left out in unfavorable conditions and had merged together, so it was very difficult to remove the yolk from the embryo. Also, many of the fin clips, collected from the female *X. birchmanni* fish, were of small samples size. When they were extracted and amplified during PCR, they did not amplify well, yielding inconclusive results or difficult analysis using Peak Scanner. If the samples did not produce favorable results when analyzed

using Peak Scanner (especially the female fin clips) they were discarded to avoid erroneous results.

Another issue which occurred while collecting the data was human error while calling the alleles. When collecting the results, there was a large amount of background noise, which made distinguishing an actual allele from the embryo or female fin clip, from a stutter difficult. The stutter seen in our data was four basepairs apart (tetrameric), which coincided with the alleles. This made it difficult to call a peak that was an actual allele and not random background noise.

To use programs such as COLONY, only two alleles could be called for each marker. However, sometimes more than two markers were called, so educated guesses had to be made to decrease the total the alleles selected, so that the analysis could be performed. Also, another problem that contributed to peak calling error was a null allele. In this instance, there was no allele visualized using the program Peak Scanner even though there should have been. In this instance, the plate did not amplify correctly during the PCR process, so fixing the error called for a repeat of this plate and an additional fragment analysis.

Some samples, which needed to be revised due to not giving good results when analyzed using Peak Scanner, had to be discarded because of a depletion of stock solution. In such cases, a decision was made to remove data on both the female and her offspring, or

just a fraction of the offspring, as there were no additional methods to collect more results due to the loss of the sample.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

The data suggests that female *Xiphophorus birchmanni* from San Pedro and Coacuilco populations' mate both multiply and at higher rates compared to other species in this genus. Previous studies done in the genus on *X. helleri* and *X. multilineatus* show that these females mate with between one to three males per brood (Simmons et al. 2008) however our data shows that female *X. birchmanni* are mating with as many as eight males per brood.

Conclusions

Female swordtails mate multiply, and there are different rates of multiple mating between the two populations. Females from the Coacuilco population are mating multiply at rates between one and four males per brood while females from San Pedro are mating at rates between one and eight males per brood. Although there could be a significant difference between the two populations, this is unlikely due to both populations dwelling at similar sites in Hidalgo, Mexico. There are no distinguishable differences between the elevation, ecology, and demography of the sites where these two populations are collected. A possible reason for the different number of multiple mates between the populations is the sample size. When running the genotypes through the program COLONY, it was noted that 23 females were analyzed from the population San

Pedro, while only 11 females were analyzed from the population Coacuilco. Increasing the sample size of Coacuilco could discern if they do mate multiply at lower rates.

Female body size and number of mates were compared against each other using a scatter plot. From the data analyzed, we can conclude there was a positive correlation between these two variables. There was also a significant relationship between these two variables ($p=0.0495$). However, our power to resolve this relationship was limited. A value of $R^2 = 0.134417$ means that the error rate was higher than expected, and that there was a low chance that the two variables would lie on the regression line (only about 13.4%).

Female body size was also compared to number of offspring and then compared using a scatter plot. From the data, it was concluded that there was also a positive correlation between these two variables ($p=0.0002$). The relationship between these two variables had a higher significance than number of mates and size of the females. The R^2 was also higher in this relationship ($R^2 = 0.394142$), showing that there was a lower error rate and the two variables had a 39.4% chance of lying on the regression line.

The GENETOP analysis revealed that two of the polymorphic microsatellite markers that were used to analyze the San Pedro samples were out of Hardy Weinberg Equilibrium. A possible reason for this could be a null allele, which is a non-amplified allele (Jones et al. 2009). Although two of the four microsatellite markers were not in

Hardy-Weinberg equilibrium, this should not affect our results since we did not study the population structure of the species.

Past behavioral studies show that females in the *Xiphophorus* genus generally prefer males with larger swords (Bosolo 1990), however male *X. birchmanni* have no sword. Behavioral studies show that females in *X. birchmanni* prefer males with no sword over males with a sword (Wong and Rosenthal 2006). Instead, females prefer large, more ornamented males (Wong and Rosenthal 2006).

Swords in the *Xiphophorus* have evolved due to a preexisting bias observed in females in the species (Rosenthal and Evans 1998). Although many male swordtails in the *Xiphophorus* species have swords, some males in the species are losing the sword ornamentation signifying females prefer other aspects on the males rather than the sword (Rosenthal and Evans 1998). *X. helleri* and *X. multilineatus* males have swords but seem to have lower rates of multiple mating. In contrast, *X. birchmanni* males do not have swords and have higher rates of multiply mating in the females. This correlation motivates future work investigating the relationship between male traits, such as swords, and female rates of multiple mating.

X. birchmanni females are mating multiply but while there is a dominant male within each brood, male fertilization success remains biased. A future prospective project would be studying high rates of multiple mating that may lead to the evolution of mechanisms

of postcopulatory sexual selection. A main focus on behavioral studies in *Xiphophorus* species is precopulatory sexual selection, such as ornamentation on the fish (Luo et al. 2005). However, from our data we can conclude that there is possible postcopulatory sexual selection occurring. Since it has been documented that females generally prefer phenotypically pleasing males such as larger brightly ornamented males, a possibility of sperm competition may exist. When females with internal fertilization mate multiply, there is an opportunity for cryptic female choice. Females may sort the sperm of her mates after mating, ultimately choosing the best possible sperm to fertilize her embryos. This possibility is consistent with our results. A future study could examine this mechanism to determine if this is a factor in the high rates of multiple mating seen in *X. birchmanni*.

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